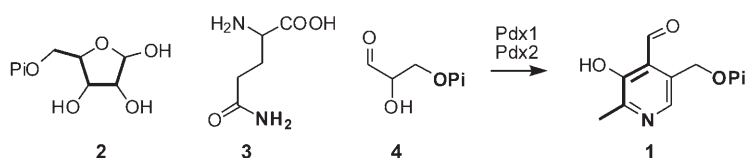


Trapping of a Chromophoric Intermediate in the Pdx1-Catalyzed Biosynthesis of Pyridoxal 5'-Phosphate**

Jeremiah W. Hanes, Ivan Keresztes, and Tadhg P. Begley*

Pyridoxal 5'-phosphate (PLP, **1**) is the biologically active form of vitamin B₆, and is essential for performing the chemistry of primary metabolism in all varieties of life. The most common pathway for the biosynthesis of PLP involves only two enzymes, Pdx1 (SNZ) and Pdx2 (SNO). This biosynthetic activity has recently been reconstituted in vitro and was shown to use ribose-5-phosphate (R5P, **2**), glutamine **3**, and glyceraldehyde-3-phosphate (G3P, **4**) as substrates (Scheme 1).^[1,2]



Scheme 1. Major biosynthetic route for pyridoxal-5'-phosphate **1**. Fragments of ribose-5-phosphate (**2**), glutamine **3**, and glyceraldehyde-3-phosphate (**4**) marked in bold are incorporated into **1**. Pi = inorganic phosphate.

The mechanistic details of this complex reaction have captured considerable recent attention owing to the interesting organic chemistry involved as well as the potential for the development of antimicrobial agents.^[3–10] The glutaminase subunit (Pdx2) generates ammonia and delivers it through a channel to the active site of the PLP synthase subunit (Pdx1).^[11–14] Biochemical studies on the Pdx1-catalyzed reaction revealed that an intermediate (I₃₂₀), formed from R5P and glutamine (or ammonia) accumulates at the active site of Pdx1.^[15,16] I₃₂₀ forms stoichiometrically with the enzyme, is covalently attached to an active site lysine, and has a high extinction coefficient (ca. 16200 M^{−1} cm^{−1} @ 320 nm).^[15] The structure of this intermediate has not yet been firmly established. Herein we report an unanticipated trapping reaction of I₃₂₀ and the structure of the trapped product.

Our previous attempts to remove I₃₂₀ from Pdx1 using either heat or acid or base denaturation failed to yield a discrete product, presumably because the released product decomposed under the harsh conditions used to remove it from the enzyme. However, we noticed that in the presence of tris(2-carboxyethyl)phosphine (TCEP), a new compound was observed by HPLC analysis of the small molecule pool. The procedure that resulted in this new species was as follows: Pdx1 was preincubated with R5P and then NH₄Cl was added to trigger I₃₂₀ formation (Figure 1A). Under these conditions, Pdx1–I₃₂₀ is stable for several hours. After purification of Pdx1–I₃₂₀ from unbound small molecules by gel filtration, the complex was incubated overnight at 15 °C in 7 M urea and 4 mM TCEP. The protein was then removed by ultrafiltration and the filtrate was analyzed by HPLC. A new species eluting at 13.2 min was observed (Figure 1B). The formation of this species (X₂₃₀) was absolutely dependent on TCEP, reconstitution of I₃₂₀, and also required protein denaturation. The absorption spectrum of X₂₃₀ is shown in (Figure 1C) and shows the loss of the 320 nm absorbance maximum of I₃₂₀.

The new compound was collected following HPLC purification and investigated by NMR spectroscopy and mass analysis. The ¹H NMR spectrum is shown in Figure 2A. From this spectrum and COSY analysis, the structure of X₂₃₀

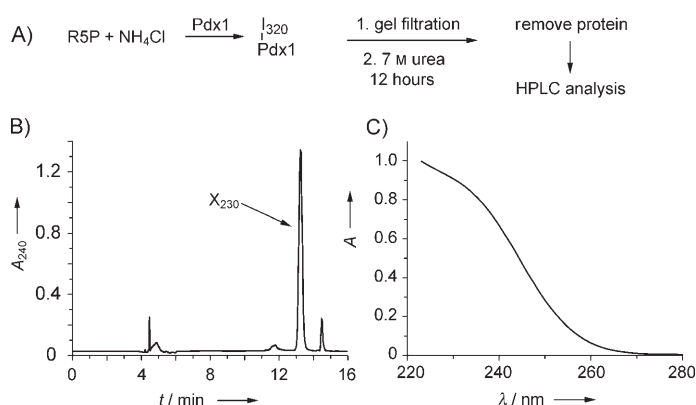


Figure 1. Trapping and isolation of I₃₂₀. A) Experimental approach for isolating I₃₂₀. After preparation, I₃₂₀ was separated from excess NH₄Cl and R5P by gel filtration and was then present in sodium phosphate buffer (50 mM, pH 7.6) with NaCl (100 mM) and TCEP (4 mM). B) HPLC analysis (detected at 240 nm) of the nonproteinaceous fraction after filtration following an overnight incubation of Pdx1–I₃₂₀ in 7 M urea in the presence of TCEP. C) UV-absorbance scan of X₂₃₀.

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Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

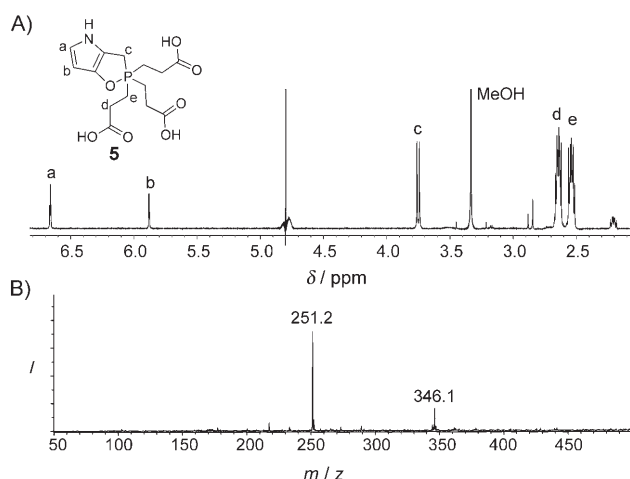


Figure 2. Structural characterization of X_{230} (**5**). A) ^1H NMR spectrum (600 MHz) of purified X_{230} in D_2O . The protons corresponding to b and c exchanged with solvent over time (b: several hours, c: several days). The solvent signal at $\delta = 4.8$ ppm was suppressed by presaturation. B) ESI-MS analysis of X_{230} performed in positive mode. The peak labeled at $m/z = 251.2$ corresponds to the +1 ion of TCEP, which is likely a fragment of the species responsible for the peak seen at $m/z = 346.1$. $m/z = 346.1$ is consistent with the +1 ion of **5**.

was tentatively assigned as **5**. ESI-MS analysis was also in good agreement with the proposed structure (Figure 2B). Although this analysis was consistent with a nitrogen-containing heterocycle, it did not directly demonstrate the presence of the nitrogen atom.

Establishing the presence of a nitrogen atom in I_{320} is of critical importance for our analysis of the mechanism of PLP formation. Previous data, obtained by high-resolution MS analysis of an I_{320} -labeled peptide from a tryptic digest, suggested that the glutamine-derived nitrogen atom was not covalently incorporated into I_{320} .^[15] However, separate experiments demonstrated that gel-purified I_{320} can be converted into PLP by the addition of G3P in the absence of any ammonia source.^[16] This suggests that the ammonia is covalently bound to I_{320} .

The isolation of X_{230} allowed us to perform a direct experiment to probe for the presence of the nitrogen atom in I_{320} . To do this, Pdx1- I_{320} formation was triggered using ^{15}N -enriched NH_4Cl , and X_{230} was purified by HPLC. The ^1H NMR spectrum obtained in 20% D_2O is shown in Figure 3A, and is consistent with structure **5**. This assignment is also supported by a variety of additional 2D NMR spectra (see Supporting Information). The large doublet of apparent triplets at $\delta = 9.8$ ppm suggested that H_a was coupled to ^{15}N . To confirm this, the proton spectrum was measured with and without broadband ^{15}N decoupling. From the pair of spectra (Figure 3B), it is clear that the heterocycle contains an ^{15}N atom that is coupled to each of the protons responsible for signals downfield of $\delta = 3.5$ ppm (a–d). As expected, the signals present in the aliphatic region of the spectrum ($\delta = 2.5$ – 2.8 ppm) were not affected by ^{15}N decoupling (e–f). This analysis demonstrates that ammonia is incorporated into I_{320}

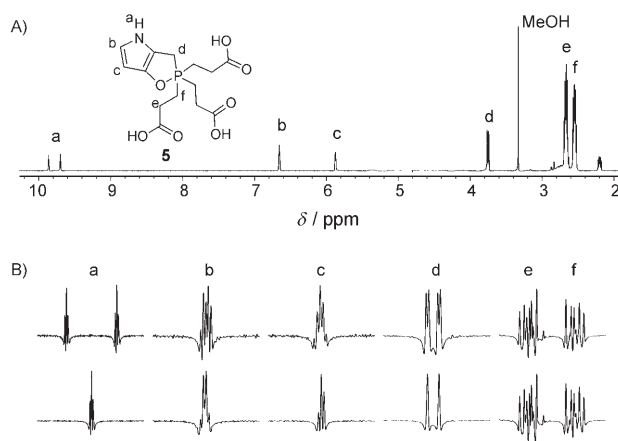


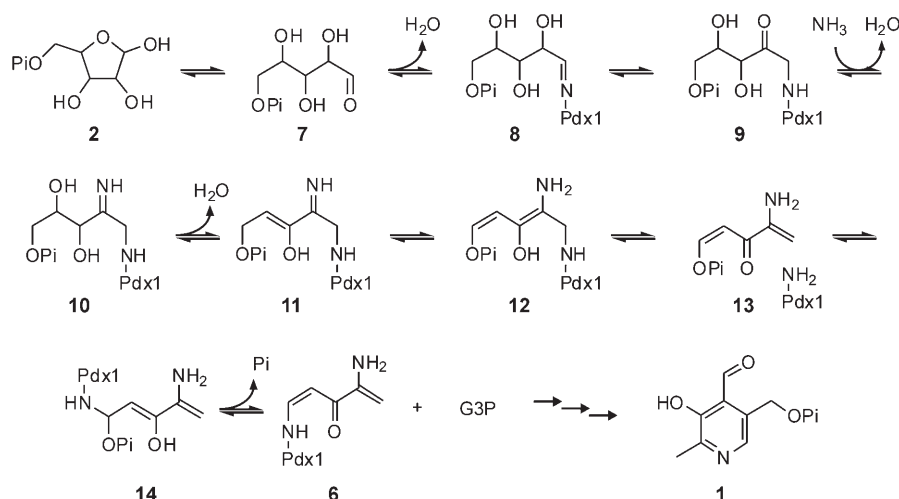
Figure 3. ^{15}N decoupling analysis of X_{230} . I_{320} was constituted using $^{15}\text{NH}_4\text{Cl}$, and then isolated according to Figure 1A. A) ^1H NMR spectrum (600 MHz) of purified I_{320} in 20% $\text{D}_2\text{O}/80\%$ H_2O (20% D_2O was used because the signals corresponding to a, c, and d exchanged with solvent over time). Processing of the data included solvent subtraction. B) Comparison of sections of the ^1H NMR spectrum shown in (A) to a spectrum obtained with broadband ^{15}N decoupling applied. Signals corresponding to a, b, c, and d were significantly simplified by ^{15}N decoupling. Processing of the data included the application of an unshifted sine-bell function prior to Fourier transformation to increase resolution.

in a covalent manner, and that X_{230} is a novel adduct of TCEP and I_{320} .

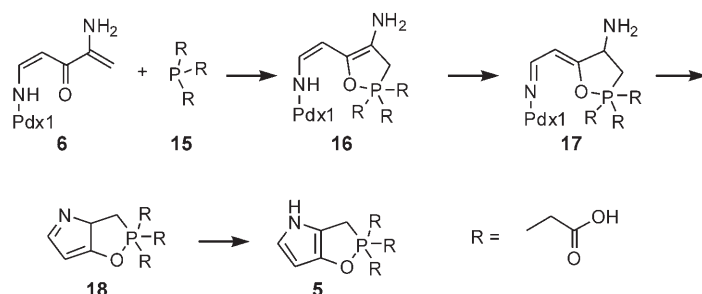
From previous work, it was shown that **6** is a likely structure for I_{320} .^[16] This proposal is based on the following: 1) I_{320} reacts with G3P to give PLP in the absence of ammonia, suggesting that ammonia is covalently bound; 2) I_{320} must be a highly conjugated system to account for its long-wavelength absorption; 3) the observation of a primary deuterium kinetic isotope effect on the formation of I_{320} using C5 pro-R deuterium-labeled suggests that one of the CH_2 protons of R5P is absent from I_{320} ; 4) phosphate elimination is stoichiometric with the production of I_{320} , demonstrating that I_{320} does not contain phosphate; and 5) analysis of the formation of I_{320} from **9** by ESI-FTMS is consistent with structure **6**. A mechanistic proposal for the formation of **6** is shown in Scheme 2.

In the absence of other substrates, R5P is ring-opened to the aldehyde **7**, and imine formation with the active-site lysine would give **8** which then rearranges to ketone **9**. This species is poised for ammonia addition at C2 to give imine **10**. This event triggers the next series of reactions leading to I_{320} . Elimination of water gives **11**, and then rearrangement to **12** occurs by deprotonation at C5. Elimination of the lysine from C1 generates **13**, and then the same lysine residue adds to C5 resulting in **14**, which facilitates phosphate elimination to give I_{320} **6**. During the normal catalytic cycle of Pdx1, the addition of G3P to I_{320} results in PLP formation in a series of reactions that are not yet well understood.

The detection of **5** as a trapped product derived from I_{320} is consistent with our assignment of structure **6** to the chromophoric intermediate. We propose (Scheme 3) that upon



Scheme 2. Mechanistic proposal for the formation of I_{320} . For compounds **2–14**, carbon atoms are defined as (right to left) C1–C5. See text for details.



Scheme 3. Proposed route for the formation of **5**. See text for details.

denaturation, TCEP (**15**) adds to **6** to give **16**, which then tautomerizes to **17** and cyclizes to **18** releasing it from the lysine residue. Aromatization gives pyrrole **5**.

The unanticipated trapping of I_{320} by TCEP demonstrates that ammonia is covalently bound to I_{320} , and supports our assignment of structure **6** to this intermediate.^[15] However, a previous MS analysis of a peptide fragment (trypsin digest) containing I_{320} suggested that it was not a nitrogen-containing compound. One explanation for this discrepancy is that **6** isomerizes upon exposure to solvent and the resulting imine undergoes hydrolysis, leading to the loss of ammonia. The current study also suggests that TCEP (and other phosphines), which is generally viewed as an inert buffer component used to protect thiols from oxidation, may have applications in the trapping of other enzyme-bound α,β -unsaturated aldehydes, ketones and imines.

Experimental Section

Procedure for the isolation of X_{230} : The overexpression and purification of *B. subtilis* Pdx1 has been described in detail.^[1,16] Following elution from the Ni^{2+} -based affinity chromatography column, the protein was dialyzed extensively against sodium phosphate buffer (50 mM, pH 7.6) containing NaCl (300 mM), TCEP (2 mM), and 25 % glycerol. The protein was then aliquoted and flash-frozen with liquid

N_2 for storage. At this point the protein concentration was approximately 2.15 mM.

To prepare X_{230} , Pdx1 (2.15 mM, 1.5 mL) was mixed with a final concentration of D-ribose-5-phosphate (1.5 mM, Sigma–Aldrich) and incubated at room temperature for 20 min. Only 1.5 mM ribose-5-phosphate was used because approximately 60 % of the active sites contain the pentose-5P as a result of copurification. NH_4Cl was added as a solid to a final concentration of 1M, and the reaction mixture was incubated for 1 hour. At this time the Pdx1- I_{320} was purified from the excess salt and small molecules using a 10DG gel filtration column (Bio-Rad) according to the product instructions. The column was pre-equilibrated with sodium phosphate buffer (50 mM, pH 7.6) containing 300 mM NaCl and 4 mM TCEP. Solid urea was then added to the protein to a final concentration of 7M and the mixture was vortexed until the urea was dissolved. The sample was then incubated for 12 h at 15 °C to allow the reaction to take place. Many products were observed if this incubation temperature was raised to 37 °C. The protein was removed from X_{230} by ultrafiltration using a 10000 kDa-cutoff Amicon Ultra-4 centrifugal filter unit at 4 °C (Millipore). X_{230} was purified by HPLC using a Supelcosil LC-18-T column (25 cm \times 10 mm, 5 μ m) equilibrated with water containing 0.1 % trifluoroacetic acid (TFA), and eluted with a gradient of MeOH containing 0.1 % TFA. The HPLC method is reported in the Supporting Information.

The major species was collected over the course of several injections, pooled, and then the solvent removed with a rotary evaporator prior to lyophilization. Following lyophilization the solid was directly dissolved in water or D_2O for MS or NMR spectroscopic analysis.

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